PURIFICATION OF FRUCTOSYLTRANSFERASE (FTase) FROM *ASPERGILLUS NIGER* TO ENHANCE PRODUCTION OF FRUCTOOLIGOSACCHARIDES (FOS) AS A FOOD ADDITIVE

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SUPERVISOR'S DECLARATION

I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology)

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In The Name of Allah, Most Gracious, Most Merciful

Love special dedicated to…

Special inspiring and special encouraging of my lovely parent: Ramli Bin Awang and Razina Binti Muhd Junus;

My siblings (long, alang, akak, kakcik, ngah, kakyah, adik),

and

also my truly best friends,

Those who has influenced my life on the right course

Thank you so much

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ABSTRACT

Nowadays, worldwide consumers are becoming increasingly aware of the relationship between food or food constituents and health. In response to an increasing demand from the consumer, fructooligosaccharides (FOS) have emerged primarily because of its functional properties rather than sweeteners. The enzyme source synthesis can be divided into two classes which are from plant and microorganism. Microorganism producing FOS from FTase had captured much attention from industrial level due to mass production and controlled environment rather than FOS produced from plant. Many researchers produced crude FTase enzyme to produce FOS. In view of that, purification of crude enzyme from microfungi is studied in order to obtain high yield of FOS. This study is carried out using molasses as a substrate in the fermentation process to produce crude enzyme from microorganism. The series of purification step will be done in order to purify the enzyme and to determine the characteristic of purified enzyme. By using an extracellular enzyme which is fructosyltransferase (FTase) from selected micro fungi, the FOS can be produced commercially from sucrose. The enzyme was able to transfer the fructosyl group from sucrose as donor producing corresponding series of FOS: 1-kestose, nystose and fructosylnystose. Although these proteins differ in their subunit structure, molecular weight, chemical susceptibility and substrate specificity, they all display both hydrolytic and transfer activities which limit the FOS production to the use of high sucrose concentration. The optimum pH and temperature for activity of FTase is between 5 to 6.5 and 50 °C to 60 °C with yield of 125 U/mL when crude FTase is used and 136.7 U/mL when purified FTase is used. For the purification studies of FTase, different micro fungi will produce different characteristic of enzyme. The enzyme was able to transfer fructosyl groups from sucrose and then catalyze the formation of short chain FOS.

ABSTRAK

Pada masa kini, para pengguna di seluruh dunia semakin sedar tentang hubungan antara makanan atau kandungan makanan dengan kesihatan. Sebagai respon kepada permintaan yang meningkat daripada pengguna, penggunaan fruktooligosakarida (FOS) telah meningkat terutamanya kerana mempunyai sifat-sifat yang berfungsi dan bukan hanya sekadar pemanis. Sumber sintesis enzim boleh dibahagikan kepada dua kelas yang terdiri daripada tumbuhan dan mikroorganisma. Mikroorganisma yang menghasilkan FOS daripada FTase telah mendapat perhatian daripada sektor perindustrian disebabkan oleh penghasilan yang berskala dan persekitaran yang terkawal berbanding dengan FOS yang dihasilkan daripada tumbuhan. Ramai pengkaji menghasilkan enzim FTase mentah untuk menghasilkan FOS. Sehubungan dengan itu, penulenan enzim mentah daripada kulat dikaji untuk mendapatkan hasil FOS yang tinggi. Kajian ini dijalankan menggunakan gula pekat yang terhasil daripada proses penapisan gula sebagai substrat dalam proses penapaian untuk menghasilkan enzim mentah daripada mikroorganisma. Beberapa siri penulenan dilakukan untuk menulenkan enzim dan untuk menentukan ciri-ciri enzim yang tulen. Dengan menggunakan enzim luar sel iaitu FTase daripada kulat yang dipilih, FOS boleh dihasilkan secara komersial dengan menggunakan sukrosa. Enzim mampu memindahkan kumpulan fructosyl dari sukrosa sebagai penderma menghasilkan siri FOS: 1-kestose, nystose dan fructosylnystose. Walaupun protein ini berbeza dalam struktur subunit mereka iaitu berat molekul, pendedahan terhadap bahan kimia dan substrat yang spesifik, mereka semua menunjukkan kedua-dua aktiviti hidrolisis dan pemindahan yang menghadkan penghasilan FOS kepada kepekatan sukrosa yang tinggi. pH dan suhu yang optimum untuk aktiviti enzim FTase antara 5 ke 6 dan 50 °C ke 60 °C dengan aktiviti sebanyak 125 U/mL bagi enzim mentah dan 136.7 U/mL apabila menggunakan enzim yang tulen. Bagi kajian penulenan FTase, kulat yang berbeza akan menghasilkan enzim dengan ciri-ciri yang berbeza. Enzim mampu untuk memindahkan kumpulan fructosyl dari sukrosa dan kemudian menjadi pemangkin kepada pembentukan rantaian pendek FOS.

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GF_2	1-kestose
GF_3	nystose
GF_4	1-β-fructofuranosylnystose
2^n	Factorial run
2n	Axial run
n_c	Center runs

LIST OF ABBREVIATION

ANOVA	Analysis of variance
CCD	Central composite design
DEAE	Diethylaminoethyl
DNS	Dinitrosalicyclic acid
FFase	Fructofuranosidase
FFT	Fructan-1-fructosyltransferase
FOS	Fructooligosaccharides
FOSHU	Food of Specified Health Used
FTase	Fructosyltransferase
OD	Optical density
PDA	Potato dextrose agar
RSM	Response surface methodology
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SST	Sucrose 1-fructosyltransferase

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

Fructooligosaccharides (FOS) are oligosaccharides of chain of fructose containing a single glucose molecule (L'Hocine et al., 2000). Fructooligosaccharides (FOS) is mainly composed of 1-kestose (GF_2), nystose (GF_3) and 1-β-fructofuranosylnystose (GF_4). They are produced by the action of enzyme fructosyltransferase (FTase) from plants and microorganisms. FOS has functional properties such as low calorific values, non-cariogenic properties, decrease level of phospholipids, triglycerides and cholesterol, help gut absorption of calcium and magnesium. These properties are useful for diabetic's products and used as prebiotics (Sangeetha et al., 2005; Sanchez et al., 2008). Fructooligosaccharides are industrially produced from sucrose by microbial enzyme with transfructosylating activity mainly found in fungi such as *Aureobasidium* sp., *Aspergillus* sp., *Arthrobacter* sp. and *Fusarium* sp. (Park et al., 2001). The enzyme source synthesis can be divided into two classes which are from plants and microorganisms. The enzyme source from plant such as sugar beet leaves, lettuce (Sangeeetha et al., 2005) and asparagus roots, onion bulbs (L'Hocine et al., 2000), FTase is produced intra- and extracellularly by several fungi. To optimize the production of FOS, the cost must be reduced and in this study, the evaluation with strain *Aspergillus niger* was carried out on molasses as a source of sucrose because pure sucrose is expensive rather than molasses (Shin et al., 2003). In view of that, purification of crude enzyme from microfungi using molasses as a substrate is studied in order to obtain high yield of FOS. The series of purification step will be done in order to purify the enzyme and to determine the characteristic of purified enzyme.

1.2 PROBLEM STATEMENT

Normally, pure sucrose is expensive and the cost to produce FTase by using sucrose is high. There are some by-product like cereal bran, corn-products, sugarcane molasses and by-products of coffee and tea processing industries were used as a substrate to produce FTase from microorganism (Sangeetha et al., 2003). This by-product can lower the cost of production of FTase. Before FOS can be produced, the enzyme FTase must be undergone a series of purification. The purification of FTase must be done in order to get high yield of FOS. Many researchers have reported the purification and characterization of FTase from various sources and FTase has been found to differ in their molecular weight and properties from one source to another (Lateef et al., 2006, Sangeetha et al., 2003).

FTase exhibits hydrolytic activity which can dominate the process (Delphine et al., 2007). This fact will lead to lower production yields and to a contamination of the final product with glucose and fructose (Delphine et al., 2007). Purification of FTase is important for batch production of FOS. By doing so, the nature of its hydrolytic activity can be studied, improve the understanding its mode of operation and be able to classified which type of enzyme should it belong to (L'Hocine et al., 2000).

1.3 RESEARCH OBJECTIVES

The main objective of this research is to purify FTase enzyme from microfungi *Aspergillus niger* isolated from bread in order to enhance the production of FOS as a food additive.

The measurable objectives are to determine:

1. The effect of temperature towards the enzyme activity.
2. The effect of substrate concentration towards the enzyme activity.
3. The effect of pH towards the enzyme activity.

1.4 SCOPE OF STUDY

Purification of crude enzyme from microfungi is studied in order to obtain high yield of FOS. This study is carried out using molasses as a substrate in the fermentation process to produce crude enzyme from microorganism. The series of purification step consist of 5 steps will be done in order to purify the enzyme and to determine the characteristic of purified enzyme. The microorganism is maintained on agar slants at 4°C. The inoculum is developed by transferring the mycelia from a 3-days old slant into the inoculum medium consist of 1% sucrose, 0.2% yeast extract at pH 5.50. The flask was incubated for 24 h at 30°C on a rotary shaker.

After 24 h, the inoculum was transferred into 100mL of fermentation medium fermentation medium consists of 17.5% w/v sucrose, 1% w/v peptone, 0.5% w/v yeast extract, 0.1% $MgSO_4.7H_2O$ and KH_2PO_4). The inoculum will undergo fermentation for 48 hours at 30 °C in incubator shaker at 250 rpm. Biomass was harvested by filtering culture broth using filter paper. The cell free fluid was taken as a source of crude extracellular enzyme.

The crude enzyme is purified and characterized based on the enzyme stability towards temperature, substrate concentration and cultivation time. The parameters are optimized by using Response Surface Methodology (RSM). Then, the activities of crude and purified enzyme are compared for FTase activity of both enzymes. The FTAse activity is analyzed by using correlation of glucose standard curve from UV-Vis Spectrophotometer.

1.5 SIGNIFICANCE OF STUDY

In Malaysia, the FTase enzyme used to be imported from other country such as Japan, India and United States (Sangeetha et al., 2005). Normally, the cost for production of FOS from commercialized enzyme is high, so by producing and purifying the enzyme, we can reduce the cost. The significance in this study is the raw material or substrate used

is molasses which is a by-product and considered as waste from sugar refinery process. Price of sucrose is quite expensive which is RM 2.30 per kilo (Norliza, A.R. 2011. Rasionalisi subsidi: Harga gula naik 20 sen esok. *Utusan Malaysia*. 9 May.) while molasses is only RM 1.50 per kilo (Husin. 2011. Molasses. http://gulamolasses.blogspot.com). In order to extend the usage of FOS, it is necessary to minimize the production cost. Molasses from sugar beet processing industry are cheap and readily available source of sucrose. By using molasses, we could reduce the cost of FTase production and concurrently we can help the sugar industry to improve their economy and manage their waste properly. By using concept of waste into wealth, molasses which is waste from sugar industry was being used to make it valuable.

Normally, crude enzyme will produce lower yield of FOS than purified enzyme. In order to fulfill high customer demand on the functional food such as FOS, the production of FOS must be increased. In order to produce high yield of FOS, the enzyme need to be purified. By doing this, the activity of enzyme will be increased and so do the production of FOS.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The production of fructooligosaccharides (FOS) has been received particular attention because of their beneficial for health and their functional properties effects. Those effects such as activation of the human immune system, resistance to infection, enhanced mineral absorption in the gastrointestinal tract, lowering of serum cholesterol and preventing carcinogenic tumors (Qiang et al., 2009). Fructooligosaccharide (FOS) has broad application in food industries due to their prebiotic properties and they are 0.4 to 0.6 times as sweet as sucrose and have been used as a functional sweetener in pharmaceutical industry (Sanchez et al., 2008). It is because of their prebiotics properties which is defined as 'non-digestible' food that beneficially affect the host by selectively stimulating the growth and activity of one or a limited number of bacteria in the colon and thus improves host health (Gibson and Roberfroid, 1995). Prebiotics also has been used to stimulate the bifidobacteria growth in the human colon (Tomasik, P.J and Tomasik, P. 2003). FOS are useful for diabetic product because of their properties such as low calorific values, non-cariogenic properties that could decrease level of phospholipids, triglycerides and cholesterol and help gut absorption of calcium and magnesium (Sangeetha et al., 2005). In order to optimize the usage of FOS, it is necessary to reduce the production cost which can be achieved by process improvement together with strain development and genetic manipulation (Shin et al., 2003). There are two classes of enzymes that are useful for FOS production at industrial scale which is fructosyltransferase (FTase) and β-fructofuranosidases (FFase) or also called invertase (Ghazi et al., 2006).

2.2 FRUCTOSYLTRANSFERASE (FTase)

FTase is an enzyme that catalyses the transformation of sucrose into fructooligosaccharides (FOS) which are important prebiotic that have a broad application in food and pharmaceutical industries (Sanchez et al., 2008). FTase catalyzes the transfer of fructosyl moieties where a donor or acceptor of these moieties can be sucrose or FOS (Antosova et al., 2002). It is widely used in food industries and pharmaceuticals industries because of their functional properties. In the industrial production of FOS, the cells with the FTase activity are produced by aerobic cultivation of fungi such as *Aspergillus aculeatus* (Nemukula et al., 2008), *Aureobasidium pullulans* (Shin et al., 2003), *Lactobacillus reuteri* (Hijum et al., 2002) and *Aspergillus niger* (L'Hocine et al., 2000). FOS is commonly present in food such as fruits, vegetables, cereals and honey. The increasing interest in prebiotic compound opens possibilities for small scale use of FTase. In recent year, the production of FOS using FTase derived from microorganism has attracted attention of many researchers (Sangeetha et al., 2005). In the industrial production of FOS, the cells with the FTase activity are produced by aerobic cultivation of fungi such as *Aspergillus niger, Aspergillus japonicus* or *Aureobasidium pullulans.* There are many researchers that reporting the optimum pH and temperature for activity of FTase between 5 to 6.5 and 50 °C to 60 °C (Ghazi et al., 2006).

2.2.1 Fructosyltransferase Mechanism

The reaction mechanism of the FTase is depending on the type of source of the enzyme. In plant and some microorganism, a series of enzyme act together whereas a single enzyme works in most of the other microorganism. For example, fructosan metabolism in plant such as Jerusalem artichoke (*H.tuberosus*) is established by two enzymes: sucrose: sucrose 1-fructosyltransferase (SST) and β (2 ⟶ 1) fructan: β (2 ⟶ 1) fructan-1-fructosyltransferase (FFT). In the first mechanism, SST converts sucrose into glucose and an oligofructoside but is unable to promote polymerization above the trisaccharide level (Yun, 1996). Further higher polymers are synthesized by FFT (Yun, 1996). The overall reaction mechanism as follows:

$$GF + GF \rightarrow GF - F + G \quad (2.1)$$

$$GF - Fn + GF - Fm\ GF - F_{n-1} + GF - F_{m+1} \quad (2.2)$$

Where GF is a sucrosyl group and n is the number of extransucrosyl fructose residues.

In the study of microbial organism mechanism, (Yun, 1996) had proposed a mathematical model for the mode of action of FTase derived from *A.pullulans*. The enzyme reaction mechanism is as follows:

$$GF_n + GF_n \rightarrow GF_{n-1} \quad (2.3)$$

Where n= 1-3

According to this mechanism, the enzyme acts on sucrose in a disproportion type reaction where one molecule of sucrose serves as a donor and another acts as an acceptor.

2.2.2 Characteristics of FTase

Several FTase have been extensively purified and characterized (Yun, 1996). The optimum pH and temperature for FTase activity is between 5 to 6.5 and 50 °C to 60 °C respectively (Lateef et al., 2006). The definition of enzyme unit also differs between researchers. Some units are defined as the amount of enzyme responsible for transferring one μmole of fructose per min while others are defined as the amount of enzyme capable of producing one μmole of glucose per min (Yun, 1996). The specificity of microbial FTase depends on the β-D-fructoside residue of sucrose (Yun, 1996). Many FOS-producing microorganisms also simultaneously produce a hydrolytic enzyme that degrades FOS (Yun, 1996). Table 2.1 summarizes the characteristic of purified enzyme from various microbial sources. The enzyme source can be divided into two classes; one is plant and the other consists of bacterial and fungal. Table 2.2 and Table 2.3 summarize the classes of enzyme.

Table 2.1: Characteristic of FTase purified from various microbial sources

Source of FTase	Purification fold	Molecular weight (kDa)	Optimum		Stability		References
			pH	Temperature	pH	Temperature	
Bacillus macerans EG-6	63.5	66	5.0	50 ℃	5.0-7.0	20 ℃ -50 ℃	Park et al., 2001
Arthrobacter oxydans J17-21	95.5	54	6.5	45 ℃	5.0-11.0	20 ℃ -40 ℃	Jang et al., 2003
Microbacterium laevaniformans ATCC 15953	4.6	64	6.0	30 ℃	5.0-7.0	-	Park et al., 2003
Aspergillus niger ATCC 20611	51.6	340	5.0-6.0	50 ℃ -60 ℃	4.5-10.0	Up to 60 ℃	Hirayama et al., 1989
Arthrobacter sp. K-1	405.3	52	6.5-6.8	55 ℃	5.5-10.0	Up to 40 ℃	Fujita et al., 1990
Streptococcus salivarus ATCC 25975	34.5	125.4	6.0-7.0	37 ℃ - 40 ℃	-	-	Song and Jacques., 1999
Microbacterium sp. AL-210	98.8	46	7.0	40 ℃	7.0-8.0	Up to 40 ℃	Cha et al., 2001
Aspergillus niger AS0023	78.5	81-168	5.8	50 ℃	4.5-11.0	30 ℃ -50 ℃	L'Hocine et al., 2000
Aspergillus foetidus	25	-	4.5	60 ℃	4.0-6.0	Up to 40 ℃	Wang and Rakshit, 2000

Source: Sangeetha et al., 2005

Table 2.2: Fructooligosaccharides enzyme from plant

Source	**Authors**
Agave Americana (agave)	Bhatia et al., 1979; Nandra and Bhatia, 1980
Agave vera cruze (agave)	Bhatia et al., 1954,1955; Satyanarayana, 1976
Asparagus officinalis (asparagus root)	Shiomi et al., 1976
Allium cepa (onion bulbs)	Darbyshire et al., 1978; Henry and Darbyshire, 1980
Cichorium intybus (chicory)	Singh and Bhatia, 1971; Chandorkar and Collins, 1972
Crinum longifolium	Bhatia et al.,1959
Sugar-beet leaves	Allen and Bacon, 1956
Helianthus tuberosus (Jerusalem artichoke)	Edelman and Dickerson, 1966; Praznik et al., 1990
Lactuca sativa L. (lettuce)	Chandorkar and Collins, 1972
Lycoris radiate (monocot)	Nagamatsu et al., 1990
Taraxacum officinale (dandelion)	Chandorkar and Collins, 1972

Source: Yun, 1996

Table 2.3: Fructooligosaccharides-producing microorganisms

Microorganisms	**Authors**
Aureobasidium pullulans	Jung et al., 1989; Yun et al., 1990; Smith et al., 1980
Aureobasidium sp.	Hayashi et al., 1989
Arthrobacter sp.	Fujita et al., 1990
Aspergillus japonicas	Duan et al., 1994
Aspergillus niger	Hidaka et al., 1989; Bealing and Bacon, 1953
Aspergillus oryzae	Pazur, 1952; Kida et al., 1988, Bealing and Bacon, 1953
Aspergillus phoenicis	Balken et al., 1991
Aspergillus sydowi	Muramatsu et al., 1988
Claviceps purpurea	Dickerson, 1972; Arcamone et al., 1970
Fusarium oxysporum	Gupta et al., 1982; Maruyama et al., 1979; Patel et al., 1994
Penicillium frequentans	Usami et al., 1991
Penicillium spinulosum	Bealing and Bacon, 1953
Phytophtora parasitica	Hankin and Meintype, 1977
Scopulariopsis brevicaulis	Takeda et al., 1994
Saccharomyces cerevisiae	Straathof et al., 1986

Source: Yun, 1996

2.3 PREBIOTICS

Prebiotics are defined as nondigestible substances of food ingredients that beneficially affect the host by selectively stimulating the growth or activity of one or limited number of bacteria in the colon that can improve the host health (Delphine et al., 2007). Prebiotic undergo fermentation by beneficial microorganism in the intestine. The

As can be inferred in Table 4.7, the computed *F test* and *Prob>F* were 3.74 and 0.0805 respectively, which implied that the model was highly significant with low probability. Results obtained adequately suggesting that the present mathematical model was in good prediction of the experimental results and as a matter of fact the terms in the model have a significant effect of the response. Moreover, the "lack of fit" value was found insignificant (Prob>F= 0.0805) which denoted that the model was desirably fit.

Fit summary output analysis indicated that the quadratic model was statistically significant to represent the enzyme activity response. The adequacy of a quadratic model was examined by *F test*, "*Prob>F*" and the determination coefficient R^2. In this case, the value of the determination coefficient for enzyme activity is 0.7709 indicate that a good agreement existed between the experimental and predicted value as well as depicting that 77.09 % of the variability in the response could be well explained by the model while 22.91 % of the total variation was poorly described by the model. The closer the R^2 is to 1, the stronger the model and the better it predicted the response (Noraziah, A.Y., 2011)